Acidithiobacillus ferriphilus sp. nov.: a facultatively anaerobic iron- and sulfur-metabolising extreme acidophile

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Abstract:	The genus Acidithiobacillus currently includes three species, A. ferrooxidans, A. ferrivorans and A. ferridurans, that conserve energy from the oxidation of ferrous iron, as well as reduced sulfur, to support their growth. Previous work, based on multi-locus sequence analysis, identified a fourth group of iron- and sulfur-oxidising acidithiobacilli as a potential distinct species. Eleven strains of "Group IV" acidithiobacilli, isolated from different global locations, have been studied. These were all shown to be obligate chemolithotrophs, growing aerobically by coupling the oxidation of ferrous iron or reduced sulfur (though not hydrogen) to molecular oxygen, or anaerobically by the oxidation of reduced sulfur coupled to ferric iron reduction. All strains were mesophilic, though some were also psychrotolerant. Strain variation was also noted in terms of tolerance to extremely low pH and elevated concentrations of transition metals. One strain was noted to be display far greater tolerance to chloride than reported for other iron-oxidising acidithiobacilli. All of the strains were able to catalyse the oxidative dissolution of pyrite and, on the basis of some of the combined traits of some of the strains examined, it is proposed that these may have niche roles in commercial mineral bioprocessing operations, such as for low temperature bioleaching of polysulfide ores in brackish waters. The name Acidithiobacillus ferriphilus is proposed for the strains described, with the type strain being M20T (= DSM 100412T,=JCM 30830T).

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Running title: *Acidithiobacillus ferriphilus* sp. nov.

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The GenBank accession numbers of the 16S rRNA gene sequences for the various strains in this study are: M20^T (KR905751), Riv13 (KR905752), PS102 (KR905753), PS104 (KR905754), PS107 (KR905755), Malay (KR905756), ST2 (KR905757), KCT10 (KR905758), KCT14 (KR905759), KCT17 (KR905760)

Abstract

The genus *Acidithiobacillus* currently includes three species, *A. ferrooxidans, A. ferrivorans* and *A. ferridurans,* that conserve energy from the oxidation of ferrous iron, as well as reduced sulfur, to support their growth. Previous work, based on multi-locus sequence analysis, identified a fourth group of iron- and sulfur-oxidising acidithiobacilli as a potential distinct species. Eleven strains of "Group IV" acidithiobacilli, isolated from different global locations, have been studied. These were all shown to be obligate chemolithotrophs, growing aerobically by coupling the oxidation of ferrous iron or reduced sulfur (though not hydrogen) to molecular oxygen, or anaerobically by the oxidation of reduced sulfur coupled to ferric iron reduction. All strains were mesophilic, though some were also psychrotolerant. Strain variation was also noted in terms of tolerance to extremely low pH and elevated concentrations of transition metals. One strain was noted to be display far greater tolerance to chloride than reported for other iron-oxidising acidithiobacilli. All of the strains were able to catalyse the oxidative dissolution of pyrite and, on the basis of some of the combined traits of some of the strains examined, it is proposed that these may have niche roles in commercial mineral bioprocessing operations, such as for low temperature bioleaching of polysulfide ores in brackish waters. The name *Acidithiobacillus ferriphilus* is proposed for the strains described, with the type strain being M20^T (=DSM 100412^T, =JCM 30830^T).

The iron-oxidising acidithiobacilli are the most widely studied of all acidophilic bacteria, due in part to their importance in environmental pollution (generation of acid mine drainage; Blowes *et al*., 2014) and mineral processing biotechnologies (Brierley & Brierley, 2013). Although it was common practice for many years to regard all Gram-negative, mesophilic chemolithotrophic acidophiles that oxidised both ferrous iron and reduced sulfur as strains of a single species (*Acidithiobacillus ferrooxidans;* formerly *Thiobacillus ferrooxidans*; Kelly & Wood, 2000), there are currently three classified species of *Acidithiobacillus* that have these core characteristics in common: *A. ferrooxidans* (Temple & Colmer, 1951), *Acidithiobacillus ferrivorans* (Hallberg *et al*., 2010) and *Acidithiobacillus ferridurans* (Hedrich & Johnson, 2013a). While iron- and sulfur-oxidising *Acidithiobacillus* spp. differ in some physiological traits (e.g. optimum and minimum pH and temperature for growth), strain variation within a single species, where reported, has sometimes been found to be as great, or greater, than differences between the type strains of each of these species.

Based on multi-locus sequence analysis (MLSA), Amouric *et al*. (2011) reported that twenty-one strains of iron-oxidising acidithiobacilli fell into four distinct clusters, each of which was proposed to be a separate species. “Group I” isolates were confirmed to be strains of *A. ferrooxidans* and “Group III” as strains of *A. ferrivorans,* both of which had been previously designated. “Group II” iron-oxidising acidithiobacilli were later classified as strains of a new species, *A. ferridurans* (Hedrich & Johnson, 2013a).

The report of Amouric *et al*. (2011) also included reference to four strains of “Group IV” iron-oxidising acidithiobacilli. Analysis of the 16S rRNA gene sequences of strains of mesophilic iron- and sulfur-oxidising chemolithotrophic acidophiles that had been isolated from different global locations and maintained within the *Acidophile Culture Collection* at Bangor University (BART-ACC; Table 1) showed that these additional strains were also more closely related to the “Group IV” bacteria than to classified *Acidithiobacillus* spp.. Several of these had been isolated from copper mines and, in one site (the Pyhäsalmi mine in Finland) they were noted to be the dominant iron-oxidising acidophiles in acidic, metal-rich waters sampled deep within the mine (Kay *et al*., 2014). Phylogenetic and physiological tests carried out with these isolates (using protocols described by Hedrich & Johnson (2013a), with all experiments replicated) has confirmed that they are strains of a distinct species, for which the binomial *Acidithiobacillus ferriphilus* is proposed.

A phylogenetic tree, showing the relationship of strains of *A. ferriphilus* to other iron-oxidising acidithiobacilli, is shown in Fig. 1. This confirmed reports (Amouric *et al*., 2011; Hedrich & Johnson, 2013a) suggesting that “Group IV” and “Group III” (*A. ferrivorans*) iron-oxidising acidithiobacilli are more closely related to each other than to “Groups I and II” (*At. ferrooxidans* and *At. ferridurans*). The fourteen strains of *A. ferriphilus* shown in Figure 1 form a tight phylogenetic cluster with >99% 16S rRNA gene sequence similarity. All the clusters were stable, and confirmed by the bootstrap analysis showing that “Group IV” separates from “Group III” which forms a separate cluster, and that these two groups cluster separately from other *Acidithiobacillus* spp..

All eleven strains of *A. ferriphilus* (ten BART-ACC strains and JCM 7812) examined in the present study were shown to catalyse the dissimilatory oxidation of ferrous iron, elemental sulfur and tetrathionate, and also the oxidative dissolution of pyrite, under aerobic conditions. All strains also catalysed the dissimilatory reduction of ferric iron under anoxic conditions, using reduced sulfur as electron donor. In addition, experiments carried out with the nominated type strain ($M20^T$) confirmed that it was able to grow anaerobically on tetrathionate via ferric iron reduction (Fig. 2), a characteristic it has in common with all other iron- and sulfur-oxidising

Acidithiobacillus spp. though not with *A. thiooxidans* which does not oxidise iron (Hallberg *et al*., 2001). None of the *A. ferriphilus* strains examined grew aerobically on hydrogen. This is also the case for most strains of *A. ferrivorans*, though all strains of *A. ferrooxidans* and *A. ferridurans* examined have been shown to grow by coupling the oxidation of hydrogen to the reduction of either molecular oxygen or ferric iron (Ohmura *et al*., 2002; Hedrich & Johnson, 2013b).

As is the case with other iron-oxidising acidithiobacilli, all strains of *A. ferriphilus* examined were strict autotrophs. They did not grow heterotrophically on organic substrates (glycerol or yeast extract) and cell numbers were similar in cultures where 20 mM ferrous sulfate medium was supplemented, or not, with either 5 mM glycerol or 0.02% (w/v) yeast extract, confirming the absence of mixotrophic growth.

The pH and temperature profiles of the eleven strains of *A. ferriphilus* examined were quite variable (Supplementary Table 1). Strain (M20^{T}) had a pH optimum and minimum for growth of 2.0 and 1.5, respectively, and a temperature optimum and maximum of 30º and 33ºC respectively (Supplementary Fig. 1). All eleven strains grew at 30ºC, but only eight at 33ºC and one (PS104) at 35ºC. All strains grew at 10ºC (five very slowly) and three strains (including the type strain) at 5ºC. From this it was concluded that *A. ferriphilus* is mesophilic, but that some strains are psychrotolerant, a feature that has only previously been reported for *A. ferrivorans* among the iron-oxidising acidithiobacilli (Hallberg et al., 2010; Liljeqvist *et al.,* 2011). All strains were acidophilic and grew at pH 1.8, though two strains did not grow at pH 1.5, and none at pH 1.25. The most acid-tolerant strain was PS104, which grew in ferrous iron medium at pH 1.35 (Supplementary Table 1). This physiological characteristic also distinguishes *A. ferriphilus* from *A. ferrivorans*, strains of which are more acid-sensitive, with the type strain having a growth pH minimum of 1.9 (Hallberg *et al*., 2010).

Osmo-tolerance was tested by growing the various strains in 20 mM ferrous iron medium (pH 1.7) containing different concentrations of magnesium sulfate. A similar approach was used to test tolerance to selected transition metals, which were also added as sulfate salts, with the exception of molybdenum where sodium molybdate was used. Oxidation of ferrous iron and increases in cell numbers were used as indicators of positive growth. Salt (sodium chloride) tolerance was tested using liquid medium (pH 2) containing 1% (w/v) elemental sulfur, and growth confirmed by monitoring culture pH (oxidation of elemental sulfur generates sulfuric acid), enumerating cells and streaking cultures identified as positive on ferrous iron overlay medium (Johnson & Hallberg, 2007) to confirm cell viability. The data obtained (Table 2) show that, although there was some variation between isolates, all eleven strains were in general highly tolerant of the cationic transition metals tested, but highly sensitive to the molybdate anion. In this respect, they were more similar to *A. ferrooxidans* and *A. ferridurans* than to the more closely related species *A. ferrivorans*, strains of which were reported to be inhibited by < 50 mM copper and < 100 mM ferric iron (Hallberg *et al*., 2010). All strains of *A. ferriphilus* were found to be particularly tolerant of ferrous iron (far more so than to ferric iron); some grew in the presence of 1 M Fe^{2+}, which is greater than values for other iron-oxidising acidithiobacilli (Hallberg *et al*., 2010; Hedrich & Johnson, 2013a). Strain variability within this novel species was again illustrated in the case of isolate KCT17, which was found to be far more sensitive to ferric iron than the ten other strains examined (Table 2).

Comparison with data from magnesium sulfate-amended cultures shows that, in many cases, tolerance of ferrous iron was limited by osmotic stress rather than by ferrous iron *per se*, with growth being observed and inhibited by the presence of similar concentrations of both magnesium sulfate and ferrous sulfate (Table 2). All eleven strains were able to grow in sulfur medium containing 250 mM sodium chloride, and two (ST2 and KCT10) in the presence of 500 mM salt, a similar concentration of chloride to that of seawater. The most salt-tolerant strain (ST2) grew in the presence of 800 mM (but not 1 M) sodium chloride in sulfur medium. However, neither strain ST2 or KCT10 grew in ferrous iron liquid medium containing 500 mM salt, even though strain ST2 had originally been isolated from the Rio Tinto on a ferrous iron overlay plate (Johnson & Hallberg, 2007) containing 500 mM sodium chloride (D.B. Johnson, unpublished). Even so, the tolerance of these two strains of *A. ferriphilus* to chloride greatly exceeded values reported for other species of iron-oxidising acidithiobacilli.

Biomass of strain M20^T was obtained by growing 10 L batch cultures in 100 mM ferrous sulfate medium at 30ºC in a bioreactor vessel (Electrolab, UK) that was stirred and aerated at ~1.5 L/min. The initial pH of the batch cultures was ~1.45, and this increased to ~ 1.75 by the time that all of the iron had been oxidised (100 mM magnesium sulfate was added to the medium in order to provide increased buffering from the bisulfate/sulfate couple). Cells were harvested, and pellets from several batch cultures combined and sent to the DSMZ (*Deutsche Sammlung von Mikrooganismen und Zellkulturen*, Braunschweig, Germany) for analysis of fatty acids, polar lipids, respiratory quinones and chromosomal base composition.

The major fatty acids found in strain M20^T grown on ferrous iron were $C_{18:1}\omega7c$, $C_{18:1}$2-OH, $C_{16:0}$ and $C_{12:0}$ With the exception of $C_{18:1}$2-OH, the fatty acids found and their relative abundances were similar to those reported for (iron-grown) *A. ferrooxidans*^{T} and (hydrogen-grown) *A. ferridurans*^{T} (Table 3; no published data are available for *A. ferrivorans*). The major polar lipids of strain M20^T were aminolipid, phospholipid and phosphatidylglycerol, and the major quinone present (94%) was Q8 (as also reported for *A. ferridurans*; Hedrich & Johnson, 2013a) with smaller amounts of Q9 (3%) and Q7 (2%). The mean base composition of the chromosomal DNA of strain M20^{T} was 57.4 mol% G+C; values reported for the type strains of other iron-oxidising acidithiobacilli are 58-59 mol % for *A. ferrooxidans* (Kelly & Wood, 2000), 58±0.02 mol% for *A. ferridurans* (Hedrich and Johnson, 2013) and 55-56 mol% for *A. ferrivorans* (Hallberg *et al*., 2010).

In summary, the eleven strains of iron- and sulfur-oxidising chemolithotrophic acidophiles described herein, together with four other strains included as “Group IV” acidithiobacilli by Amouric *et al*. (2011), are representatives of the novel species, *A. ferriphilus*. Although more closely related (from MLSA analysis) to *A. ferrivorans* than to either *A. ferrooxidans* or *A. ferridurans*, strains of *A. ferriphilus* share some traits with the former, and others with the latter two species. Some physiological characteristics suggest that some strains of *A. ferriphilus* could play a significant role in commercial mineral bio-processing operations, such as low temperature bioleaching of polysulfide ores in brackish waters, where they would, in theory, be superior to other species due to the unique combination of transition metal-, salt- and psychro-tolerance.

Description of *Acidithiobacillus ferriphilus* sp. nov.

Acidithiobacillus ferriphilus (fer.ri'phi.lus. L. n. ferrum iron; N.L. adj. *philus -a -um* (from Gr. adj. *philos -ê -on*) friend, loving; N.L. masc. adj. *ferriphilus* iron-loving, referring to its ability to grow in the presence of elevated concentrations of ferrous iron).

Gram-negative, motile, straight rods (1 to 2 μm long) that do not form endospores. Forms small, ferric iron-stained colonies on acidic ferrous iron overlay media. Obligate chemolithoautotroph, capable of growth using ferrous iron or reduced sulfur (elemental sulfur or tetrathionate) as electron donors. Facultative anaerobe, capable of coupling the oxidation of ferrous iron and reduced sulfur to the reduction of molecular oxygen, and the oxidation of reduced sulfur to the reduction of ferric iron. Mesophilic and extremely acidophilic, though some strains are psychrotolerant (and grow at 5ºC). The type strain has pH and temperature growth optima of 2.0 and 30˚C, respectively. The G + C content of the chromosomal DNA of the type strains is 57.4 mol%.

The type strain, M20^{T} (=DSM 100412^{T}, =JCM 30830^{T}) was isolated from an acidic pool in a geothermal area of Montserrat (West Indies). Other strains of *A. ferriphilus* have been isolated from acidic iron-rich waters at metal mine sites.

Acknowledgement

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Table 1. Sites of origin of the various strains of *A. ferriphilus* used in the present study

Strain	Source	Country	Reference
M20^T	Galway's Soufriere	Montserrat (West Indies)	Atkinson *et al*. (2000)
Riv13	White River		
JCM 7812	Sulfur/iron sulfide mine	Japan	Wakao *et al*. (1991)
Malay	Metal mine drainage water	Malaysia	D.B. Johnson (unpublished)
ST2	Rio Tinto	Spain	D.B. Johnson (unpublished)
PS102	Copper/zinc mine	Finland	Kay *et al*. (2014)
PS104			
PS107			
KCT10	Copper mine, Utah	USA	D.B. Johnson (unpublished)
KCT14			
KCT17			

Table 2. Comparison of tolerance of strains of *A. ferriphilus* to elevated concentrations (millimoles/L) of selected transition metals, magnesium and sodium chloride. Numbers indicate minimum inhibitory concentrations and (in parentheses) the maximum concentrations at which growth was observed

Strain	Fe(II)	Fe(III)	Co	Cu	Mo	Ni	Zn	Mg	NaCl*
M20^T	1000 (900)	500 (300)	600 (400)	500 (300)	< 0.1	500 (300)	800 (700)	1000 (900)	500 (250)
JCM 7812	700 (500)	500 (300)	600 (400)	300 (100)	< 0.1	500 (300)	800 (700)	900 (800)	500 (250)
Malay	1200 (1000)	500 (300)	600 (400)	300 (100)	< 0.1	500 (300)	800 (700)	1200 (1000)	500 (250)
Riv13	1200 (1000)	500 (300)	600 (400)	300 (100)	< 0.1	500 (300)	700 (600)	1200 (1000)	500 (250)
ST2	1200 (1000)	500 (300)	600 (400)	500 (300)	< 0.1	500 (300)	800 (700)	1200 (1000)	1000 (800)
PS102	900 (700)	300 (100)	600 (400)	500 (300)	< 0.1	500 (300)	800 (700)	900 (800)	500 (250)
PS104	1200 (1000)	500 (300)	800 (600)	500 (300)	< 0.1	500 (300)	800 (700)	1200 (1000)	500 (250)
PS107	1000 (900)	500 (300)	400 (200)	300 (100)	< 0.1	300 (100)	800 (700)	1200 (1000)	500 (250)
KCT10	1000 (900)	300 (100)	600 (400)	500 (300)	< 0.1	500 (300)	800 (700)	1000 (900)	700 (500)
KCT14	1200 (1000)	500 (300)	600 (400)	500 (300)	< 0.1	500 (300)	800 (700)	1200 (1000)	500 (250)
KCT17	700 (500)	100 (50)	600 (400)	500 (300)	< 0.1	300 (100)	600 (400)	1200 (1000)	500 (250)

*grown on elemental sulfur; all other data refer to cultures grown on ferrous iro

Table 3. Cellular fatty acids (shown as percentage values) in *A. ferriphilus* strain M20^T grown on ferrous iron at pH 1.45-1.75 and 30ºC, and comparison with values reported for the type strain of *A. ferrooxidans* and *A. ferridurans*. No published data are available for *A. ferrivorans*

Fatty acid	*A. ferriphilus* M20^T	*A. ferrooxidans* ATCC 23270$^{T(1)}$	*A. ferridurans* ATCC 33020$^{T(2)}$
$C_{12:0}$	5.7	8	6.6
$C_{13:}$ AT12-13	0.4	11	0.3
$C_{14:0}$	0.2	-	0.7
$C_{15:0}$	-	-	0.5
$C_{15:0}$ 3-OH	-	-	-
$C_{16:0}$	7.5	$18^{\#}$	15.6
$C_{16:0}$ 2-OH	0.5	-	1.2
$C_{16:0}$ 3-OH	2.7	-	0.9
$C_{16:1}$	-	$21^{§}$	-
$C_{16:1}$ ω5c	0.4	-	-
$C_{17:0}$	0.5	$6^{¥}$	1.9
$C_{17:0}$ cyclo	-	-	6.7
$C_{17:0}$ 2-OH	0.1	-	-
$C_{17:1}$ ω6c	0.4	-	-
$C_{17:1}$ ω8c	0.6	$0.5^{\pm}$	0.7
$C_{18:0}$	0.9	0.5^{**}	1.5
$C_{18:0}$ 2-OH	0.5	-	-
$C_{18:0}$ 3-OH	0.1	-	-
$C_{18:1}$ ω5c	-	-	0.6
$C_{18:1}$ ω7c	33.8	$21.5^{‡}$	16.6
$C_{18:1}$ 2-OH	10.3	-	0.9
11 methyl $C_{18:1}$ ω7c	-	-	0.3
$C_{19:0}$ 10 methyl	1.0		
$C_{19:0}$ cyclo ω8c	-	14.5	17.5
$C_{20:2}$ ω6,9c	-	-	0.4
Summed feature 1*	-	-	0.3
Summed feature 2*	10.14	-	9.9
Summed feature 3*	21.57	-	14.9

$^{(1)}$grown on ferrous iron at pH 1.5 and 25ºC (Mykytczuk *et al*., 2010); $^{(2)}$grown on hydrogen at pH 2 and 30ºC (Hedrich and Johnson, 2013).

* Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 1 contains iso-$C_{15:1}$ and/or iso-$C_{13:0}$ 3-OH.; summed feature 2 contains $C_{14:0}$ 3-OH and/or iso-$C_{16:1}$; summed feature 3 contains $C_{16:1}$ω 7c, $C_{16:1}$ω6c and/or iso-$C_{15:0}$ 2-OH. $^{\#}$value represents $C_{16:}$

$_{0,}$ $C_{16:0}$ 2-OH, $C_{16:0}$ 3-OH; [§]value represents $C_{16:1}$ ω6c, $C_{16:1}$ 2-OH, $C_{16:1}$ ω5c; [¥]value represents $C_{17:0}$, $C_{17:0}$ cyclo, $C_{17:0}$ 2-OH; [±]value represents $C_{17:1}$ ω8c, $C_{17:1}$ ω6c, $C_{17:1}$ anteiso; **value represents $C_{18:0}$, $C_{18:0}$ 2-OH, $C_{18:0}$ 3-OH; [‡]value represents $C_{18:1}$ ω7c, $C_{18:1}$ 2-OH

Fig. 1. Neighbour-joining phylogenetic tree derived from 16S rRNA gene sequence data showing the relationship of strain M20^T and other *A. ferriphilus* (“Group IV”) strains (in bold for strains used in the present study) to other *Acidithiobacillus* spp.. Topologies of trees constructed by parsimony and maximum-likelihood algorithms were similar. GenBank accession numbers are given in parenthesis for each strain, and the tree was rooted with iron-oxidizing acidophile *Acidiferrobacter thiooxydans* (AF387301). Bootstrap values are given at the respective nodes and the scale bar represents 0.002 % sequence divergence

Fig. 2. Correlation between cell numbers and ferric iron reduced in cultures of isolate M20^T grown anaerobically on tetrathionate as electron donor and ferric iron as electron acceptor (r^2 = 0.71)

Figure 1

Click here to download Figure Fig. 1.pptx

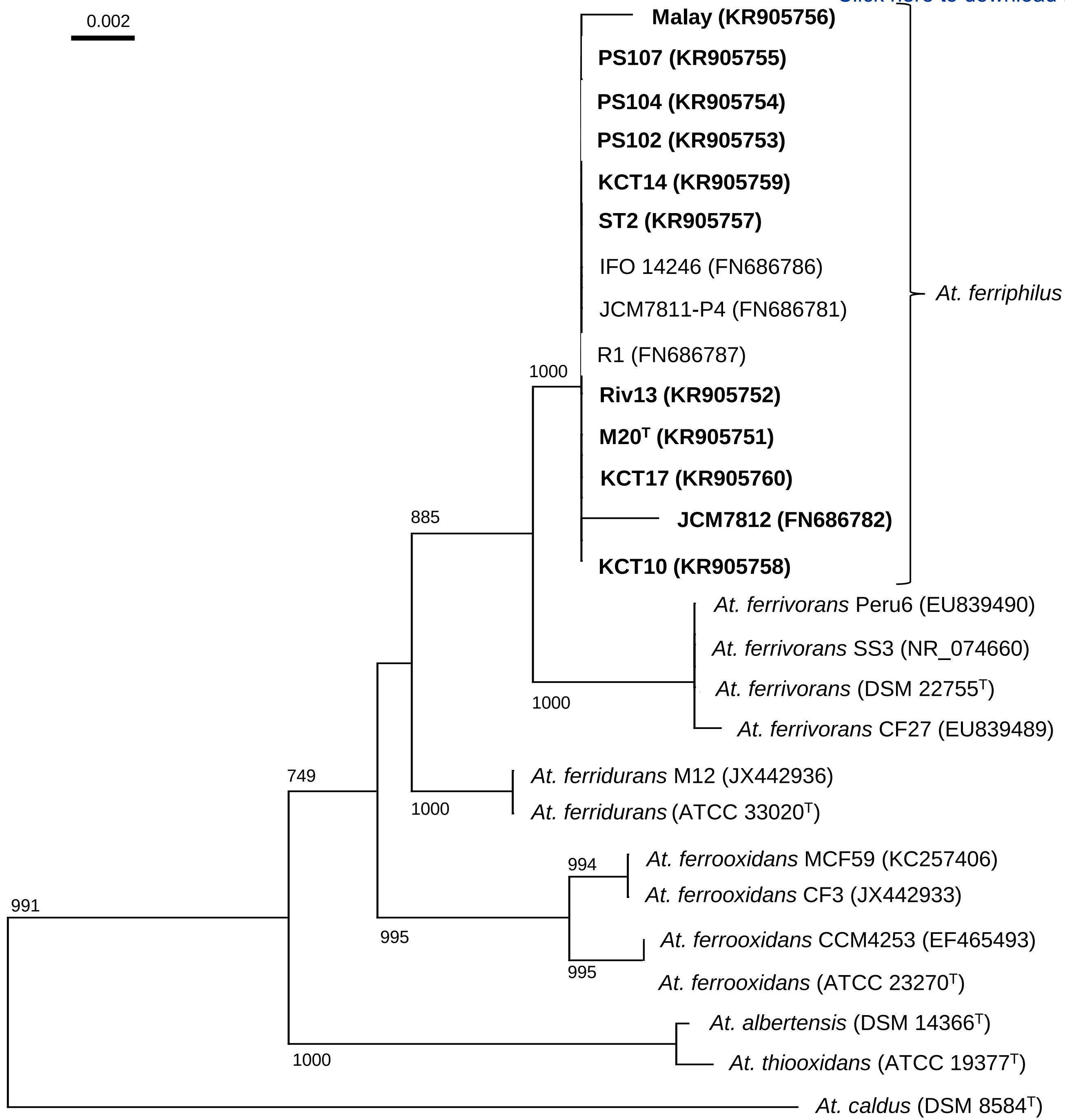
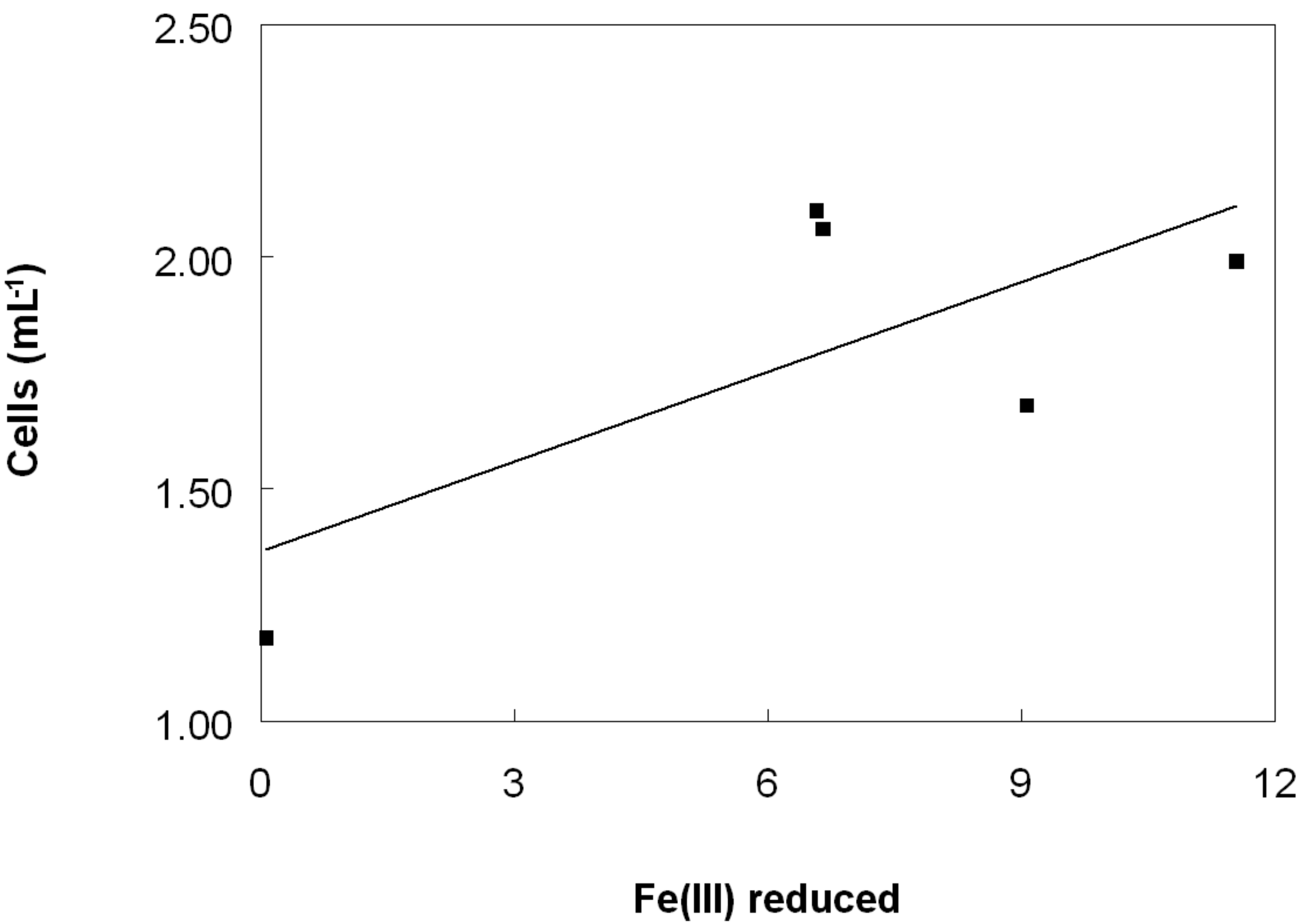


Figure 2



Acidithiobacillus ferriphilus sp. nov.: a facultatively anaerobic iron- and sulfur-metabolising extreme acidophile

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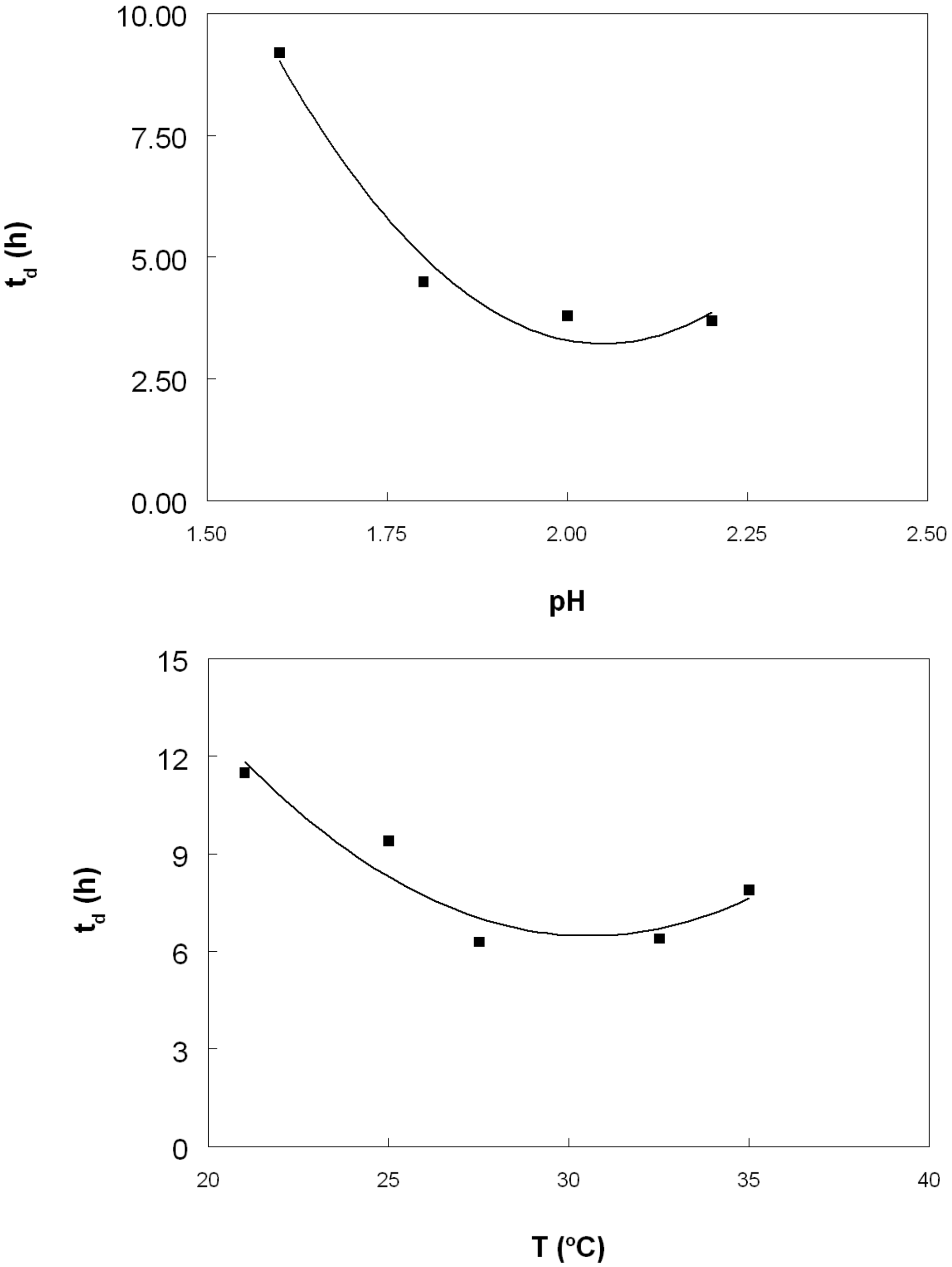
Supplementary files

Supplementary Table 1. Physiological characteristics of strains of *A. ferriphilus*

Strain	**Oxidation of**				**Reduction of**	**Growth on**			**Growth at**					
	Fe(II)	FeS_2	S°	S_4O_6	Fe(III)	H_2	YE	Glycerol	5°C	10°C	33°C	35°C	pH 1.25	pH 1.5
M20^T	+	+	+	+	+	-	-	-	+	+	+	-	-	+
JCM 7812	+	+	+	+	+	-	-	-	-	+	+	-	-	-
Malay	+	+	+	+	+	-	-	-	+	+	+	-	-	+
Riv13	+	+	+	+	+	-	-	-	-	+	+	-	-	+
ST2	+	+	+	+	+	-	-	-	+	+	+	-	-	+
PS102	+	+	+	+	+	-	-	-	-	±	+	-	-	+
PS104	+	+	+	+	+	-	-	-	-	±	+	+	- (+ at pH 1.35)	+
PS107	+	+	+	+	+	-	-	-	-	±	-	-	-	+
KCT10	+	+	+	+	+	-	-	-	-	±	±	-	-	+
KCT14	+	+	+	+	+	-	-	-	-	+	-	-	-	-
KCT17	+	+	+	+	+	-	-	-	-	±	-	-	-	+

Growth was assessed by monitoring cell numbers, and oxidation/reduction of iron or acid-production (sulfur cultures)



Supplementary Fig. 1. The effect of (top) pH, and (bottom) temperature on mean generation times (t_d) of strain M20^T. Each data point represents a t_d value obtained from a series of data obtained for each condition used